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ABSTRACT: Orexin is a hypothalamic neuropeptide that regulates feeding behavior in rats. Orexin-B has recently been cloned in pigs and was shown to stimulate food intake after intramuscular injection. This study was designed to determine whether intracerebroventricular (ICV) and intravenous injections of orexin could regulate appetite in sheep. Suffolk wethers were moved to indoor facilities, adapted to diets for 6 wk, and trained to stand in stanchions for 3 to 6 h each day for 2 wk before indwelling ICV cannulas were installed. These sheep were provided water and they consumed feed ad libitum. On the day before an experiment, each sheep was cannulated in a jugular vein. On the day of an experiment, sheep were placed in stanchions and allowed to stand for 1 h before use. Sheep were then monitored over a 2-h control period before i.v. injection with saline or porcine orexin-B (3 μg/kg BW) or ICV injection with artificial cerebrospinal fluid (CSF), orexin (0.03, 0.3, or 3 μg/kg BW) or in a second experiment with either orexin B (0.03, 0.3, 3 μg/kg BW), neuropeptide-Y (NPY; 0.3 μg/kg BW), or orexin plus NPY. Food intake was monitored for consecutive 2-h periods. The i.v. injections of orexin did not affect food intake or metabolite or hormone concentrations. In ICV sheep, orexin increased food intake at 2 ($P < 0.04$) and at 4 h ($P < 0.02$). Food intake was greatest with the 0.3 μg/kg BW dosage of orexin ($P < 0.05$). In the first 2 h after injection, orexin had an effect similar to that of NPY (0.23 kg for orexin and 0.2 kg for NPY). The combination of NPY and orexin had a greater effect on food intake (to 0.34 kg) than did either orexin ($P < 0.05$) or NPY ($P < 0.008$) alone. Differences were not apparent in the subsequent 2-h interval. No differences were noted in free fatty acid, glucose, growth hormone, luteinizing hormone, or insulin concentrations following orexin injection. There was an effect of ICV orexin treatment on plasma cortisol concentrations ($P < 0.002$). Cortisol was increased by orexin at the 0- to 2-h ($P < 0.008$) and in the 2- to 4-h ($P < 0.009$) intervals after orexin injection. These data indicate that central administration of orexin stimulates feed intake in sheep.

Key Words: Appetite, Hydrocortisone, Hypothalamus, Sheep

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Introduction

The hypothalamus plays a major role in regulation of appetite. Central to this regulation is the recent discovery of a new class of appetite regulatory peptides called *orexins* (Sakurai et al., 1998). Orexin neurons have been identified in the rat hypothalamus (Cai et al., 1999; van den Pol et al., 1998), where orexin mRNA levels are increased with fasting (Sakurai et al., 1998), suggesting that orexin release is associated with appetite control. Intracerebroventricular (**ICV**) injection of orexin-B caused an increase in food intake in rats but had no effect when injected into hypothalamic nuclei that regulate appetite (Sweet et al., 1999). The injection of orexin antibodies inhibited food intake (Yamada et al., 2000), and leptin injections inhibited orexin mRNA levels, suggesting a role for orexin in mediating leptin effects on appetite (Beck and Richy, 1999).

Lubkin and Stricker-Krongard (1998) have postulated a role for orexin in endocrine and metabolic regulation. Indeed, orexin axons in the rat project to the suprachiasmatic nucleus, paraventricular nucleus, arcuate nucleus, and other endocrine regulatory areas (Date et al., 1999). Orexin has been shown to regulate luteinizing hormone in ovariectomized rats (Pu et al., 1998; Tamura et al., 1999). In addition, orexins stimulate in vitro corticosterone release (Malendowics et al., 1999), stimulate drinking behavior (Kunii et al., 1999), increase catecholamines (Shirasaki et al., 1999), increase heart rate (Shirasaki et al., 1999), and regulate sleep (Lin et al., 1999).

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Recently, Dyer et al. (1999) cloned porcine orexin and examined the effects of orexin-B on voluntary food intake. Porcine orexin-B has 96% homology to human orexins. Intramuscular injections of orexin-B produced an 18% increase in 24 h feed intake. The current study was initiated to determine whether ICV or i.v. injection of porcine orexin-B would stimulate appetite and alter endocrine function in sheep.

Materials and Methods

Castrated male sheep (9 mo old, 43 ± 4.1 kg; n = 4) were used in these studies. Sheep were housed two per pen in a temperature- and light-controlled facility. Animals were fed a feed concentrate that contained 12% protein and was calculated to meet 100% of daily requirements. Feed was given twice daily at a total of 4% BW. Small amounts of hay were provided daily but not during a food intake study. Stainless steel cannulas were surgically implanted in the lateral cerebral ventricle as previously described (Sartin et al., 1988; McMahon et al., 1999) and sheep were allowed to recover for 2 wk before placement of cannulas was confirmed by ventriculography. After two additional weeks, sheep were used in the experiments (lasting from December through March). Each treatment was administered to each sheep with at least a 1-wk interval between treatments. The order of treatments was randomized. After an ICV injection experiment, sheep were treated with a single i.m. injection of tetracycline to prevent infection.

On the day before an experiment, sheep were fitted with jugular vein cannulas. On the morning of an experiment, sheep were fed at 0600 and then placed in stanchions at 0730 with 1.8 kg of food available plus water. After 1 h, 1.8 kg of new food was presented and blood sample collection begun (3 mL per sample at 15-min intervals for 6 h). After the first 2 h, new food (1.8 kg) was presented and either 200 μL of artificial CSF (McMahon et al., 1999) or porcine orexin-B (0.03, 0.3, or 3 μg/kg body weight) was injected into the lateral ventricle over a 15-s duration. In another experiment using the protocol described above (the same animals were used as in the above experiments), artificial CSF, porcine orexin (0.3 μg/kg BW), ovine neuropeptide-Y (**NPY**) (0.3 μg/kg BW; Sigma Chemical Company, St. Louis, MO), or orexin plus NPY were injected into the lateral ventricle. Food intake was determined by weighing the food not consumed at 2-h intervals (–2 to 0, 0 to 2, 2 to 4 h).

Based on an effect of orexin on food intake at the 0.3 μg/kg dose, plasma was assayed for samples collected from animals treated with only this optimal dose of orexin. The NPY and NPY+orexin groups were examined for food intake but not for metabolites or hormones. Plasma samples were analyzed for glucose and free fatty acids using previously validated kits (Sartin et al., 1988; McMahon et al., 1999). Hormones (GH, cortisol, insulin, LH) involved in the regulation of these metabolites or thought to be regulated by appetite regulatory peptides were determined by RIA (Sartin et al., 1988; Thompson et al., 1994; McMahon et al., 1999). The intra- and interassay CV for the assays were 2.9% and 17% for cortisol, 7.8% and 12.2% for GH, and 5.8% and 14.5% for insulin. The intraassay CV for LH was 7.4%.

These experiments were repeated using i.v. injections of orexin-B (0, 0.3, 3 μg/kg BW). Blood samples and food intake were as described above.

Data from the first experiment were analyzed using incomplete randomized block factorial designs with time and treatment regarded as fixed effects and sheep as block. Tukey's test or orthogonal contrasts were used to test for specific differences in treatment effects. Data from the second experiment were analyzed using randomized complete block designs for food intake at each of the two time points (Rao, 1998). A *P*-value of 0.05 was considered statistically significant.

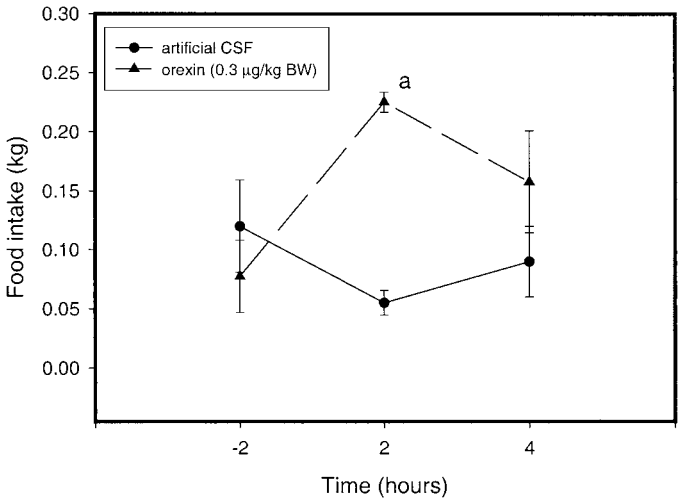

Figure 1. Effect of intracerebroventricular injection of artificial cerebrospinal fluid (CSF) or orexin-B (0.3 mg/kg BW) on food intake at each 2-h interval; the letter *a* indicates significant differences from controls, $P < 0.05$.

Results

The ICV injection of orexin produced an increase in short-term food intake at 2 h ($P < 0.05$), which had returned to an intake similar to that of controls from 2 to 4 h (Figure 1). Cumulative food intake differed at 2 h ($P < 0.04$) and 4 h ($P < 0.03$) after the injection (Figure 1). During the 0- to 2-h and 2- to 4-h periods after injection, the 0.3-μg dose of orexin increased food intake ($P < 0.05$), but there was no dose-response effect of orexin (Figure 2). There were significant orexin and NPY effects at the 0 to 2 ($P < 0.0002$) but not the 2- to 4-h interval ($P = 0.16$; Figure 3). In addition, the NPY-plus-orexin-treated sheep consumed more food than either the orexin- ($P < 0.04$) or NPY- ($P < 0.01$) treated sheep.

Means of metabolites and hormones were examined for the same time intervals as feed intake. There was

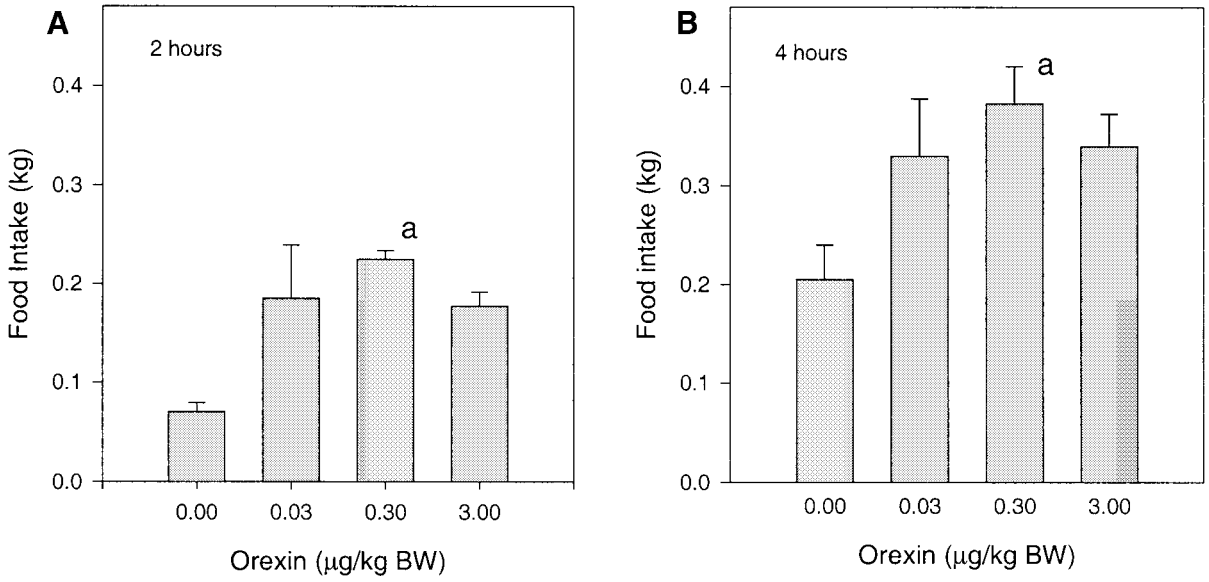

Figure 2. Effect of intracerebroventricular injection of orexin-B on cumulative food intake in sheep (graphed using 2-h interval data from Figure 1). Panel A is food intake during h 0 to 2 after injection. Panel B is cumulative food intake 4 h after injection. There was a treatment effect of orexin at 2 h ($P < 0.04$) and 4 h ($P < 0.02$). The letter *a* indicates different from control, $P < 0.05$.

an effect of treatment on cortisol ($P < 0.002$). Cortisol concentrations differed between treatment groups at both the 0- to 2-h interval ($P < 0.002$; control, 19.5 ± 3.4 vs orexin, 37.4 ± 4.8 nmol/L) and the 2- to 4-h interval ($P < 0.009$; 28.0 ± 4.3 vs 43.6 ± 5.6) (Figure 4). There were no treatment effects on plasma glucose, FFA, insulin, GH, or LH concentrations. Orexin treatment means ± SEM for each 2-h period (–2 to 0 h; 0 to 2 h; 2 to 4 h) were as follows: for FFA, 0.046 ± 0.002, 0.051 ± 0.003, 0.043 ± 0.001 mEq/L; for glucose, 70.3 ± 1.3, 73.0 ± 1.4, 74.0 ± 1.5 mg/100 mL; for insulin, 111.3 ± 4.4, 115.3 ± 4.9, 119.0 ± 4.8 nmol/L; for GH, 0.77 ± 0.07, 1.25 ± 0.3, 1.32 ± 0.15 ng/mL (saline controls also increased with time); and for LH, 1.03 ± 0.01, 1.03 ± 0.12, 1.2 ± 0.18 ng/mL.

The i.v. injection of orexin had no effects on food intake. Means for cumulative food intake (n = 5) for 0- to 2-h and 2- to 4-h for saline-injected control sheep were 0.05 ± 0.05 kg and 0.18 ± 0.09 kg. Means for orexin-

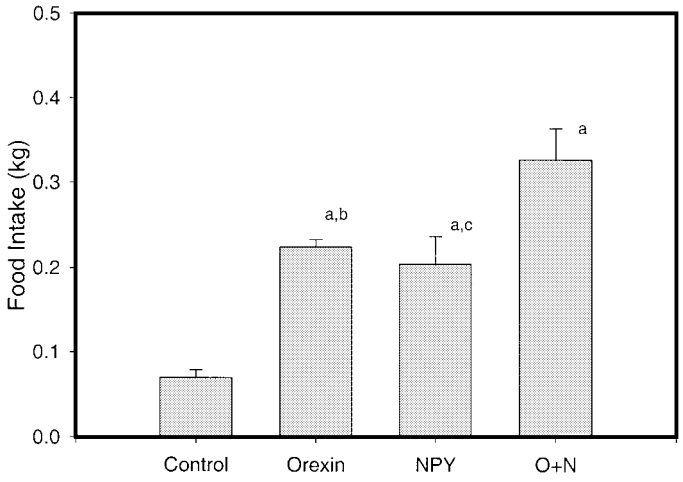

Figure 3. Effects of intracerebroventricular injection of orexin-B (O; 0.3 μg/kg BW), neuropeptide-Y (NPY; 0.3 μg/kg BW) and O plus NPY on food intake in sheep 2 h after injection; n = 4 per group; a differs from control, $P < 0.05$; b differs from O + NPY ($P < 0.04$); c differs from O + NPY ($P < 0.007$).

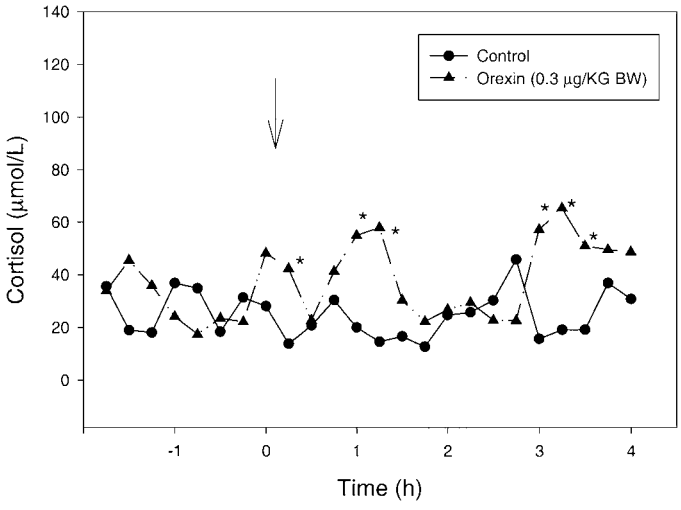

Figure 4. Effect of intracerebroventricular injection of orexin on plasma cortisol concentrations. There was a significant effect of treatment ($P < 0.001$). The symbol indicates different from control ($P < 0.05$). Arrow indicates the time point when artificial CSF or orexin were injected. The pooled standard error of the mean is 11.2.

(3 μg/kg BW) treated sheep were 0.14 ± 0.09 and 0.19 ± 0.11 kg.

Discussion

Orexin has been demonstrated to regulate appetite in rodents when infused into cerebral ventricles and in swine when injected intramuscularly. Our data indicate that a bolus injection of orexin-B can stimulate an increase in appetite when administered into the ventricles at a dose similar to those for NPY in sheep (Miner et al., 1989). Moreover, the transient nature of the response is as that described for rodents, although the effect was of lower duration when compared with the ICV infusion of orexin in rodents (Sakurai et al., 1998). As further, although subjective, evidence for an effect of orexin in these sheep, we observed an increase in the hematocrit following the orexin-induced ingestion of feed (this was based on visual comparison of centrifuged blood samples from orexin and control sheep and not a quantitative measurement). This was, in turn, followed by a prolonged period of water ingestion (water intake was not measured) when compared with drinking behavior in saline-treated sheep. Following the water ingestion, the hematocrit was reduced. This is interesting in light of data indicating that orexin can regulate drinking behavior in rodents (Kunii et al., 1999). Miner et al. (1989) also reported that NPY induced drinking behavior in sheep, although this effect was not seen in our experiments.

There were no obvious differences in the food intake responses between the presently tested doses of orexin and NPY (positive control). In rats, NPY was found to be more potent than either orexin-A or -B. Orexin-A was in turn described as having a consistent effect, compared with orexin-B, which was only occasionally effective (Edwards et al., 1999). It is interesting that NPY and orexin combined had an effect that was more prominent and persistent than the individual effects of these agents. In rodents, it seems that NPY and orexin neurons form synapses on one another and may interact to regulate appetite (Horvath et al., 1999). Thus, the combined dose of orexin and NPY may be enhanced by producing a direct action on appetite plus an effect due to activation of NPY and orexin neurons.

Our observation that i.v. administration of orexin had no effect on food intake is consistent with the study demonstrating that orexin-B does not cross the blood-brain barrier of rodents (Kastin and Akerstrom, 1999). However, doses of orexin-B at 100 times higher than our highest doses injected intramuscularly into pigs could activate food intake, suggesting that higher doses infused intravenously might have been successful.

Another appetite regulator, NPY, has effects on endocrine regulation in several species. For example, NPY regulates LH release in rodents and sheep (Jain et al., 1999; McShane et al., 1992) and is an inhibitor of GH release in rats (Rettori et al., 1990). We have observed the opposite effects on GH regulation in the sheep (McMahon et al., 1999) following ICV injection and subsequent infusion of NPY. This major species difference, as well as studies that suggest orexin's connections to hypothalamic areas regulating endocrine function, prompted our investigation of orexin in endocrine and metabolic regulation in sheep. However, our data do not indicate effects of ICV orexin on GH, insulin, LH, glucose, or FFA. There does appear to be a pulse of FFA after orexin injection. When compared against the saline control, the data were not significant. Due to the short period of sampling, pulse parameters were not determined for LH, but there were no effects on mean LH concentrations after ICV orexin injection. This is at variance with data for rats demonstrating an effect of similar doses (0.3 and 3 nmol) of ICV orexin on LH release (Pu et al., 1998; Tamura et al., 1999). Although the stimulatory effect of orexin was described for estrogen-progesterone primed, castrated female rats (Pu et al., 1998), orexin was inhibitory in castrated, unprimed female rats (Pu et al., 1998; Tamura et al., 1999). Based on our data, orexin may not be active in castrated male sheep, at least at the doses tested in this study.

It is interesting to note that NPY is present in the neurons innervating the adrenal cortex and that NPY can increase the release of aldosterone and catecholamines (Renshaw et al., 2000). In vitro data demonstrate that orexin can likewise activate corticosterone synthesis and release from the rat adrenal cortex (Malendowicz et al., 1999). In addition, s.c. injections of orexin-A, and to a lesser extent orexin-B, can increase plasma levels of corticosterone. In the present study, cortisol secretion (in vivo) was not altered by i.v. injection of orexin. However, ICV injections of orexin produced a modest increase in cortisol in our sheep, suggesting a central mechanism for the regulation of cortisol rather than a peripheral action. The cortisol response was not quantitatively great and occurred 30 min after ICV orexin injection. At this time, our data are suggestive of orexin's increasing plasma cortisol concentrations. Perhaps a different design with higher doses or a bolus ICV injection followed by infusion of orexin might be more valuable in delineating this effect of orexin.

Our data indicate that orexin-B is a potent regulator of food intake in sheep. Although orexin neurons innervate the arcuate nucleus, there is no clear evidence for a major endocrine role of this peptide in sheep. More detailed studies need to be performed to fully explore this possibility.

Implications

In spite of the importance of food intake regulation to animal production, neural regulation of food intake has not been extensively studied in farm animals. This study has determined that a new peptide, orexin, can activate central nervous system mechanisms to increase food intake in sheep. Orexin is an endogenous regulator of appetite in rodent models. Because orexin is found to regulate food intake in sheep, it may also

serve as an endogenous appetite regulator in this species.

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